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FOREWORD

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INTRODUCTION

In eukaryotes, the ends of linear chromosomes are protected from damage and rearrangements by a complex of G-rich repetitive sequences and protein, called telomeres. Replication and maintenance of telomeres is necessary for normal cellular proliferation. In most eukaryotes, the reverse transcriptase telomerase is responsible for replicating the end of a chromosome and thus maintaining telomere length. Absence of telomerase activity results in progressive telomere shortening with continued cell division. When telomeres are too short to maintain function, most cells stop dividing. Loss of viability with continued propagation of telomerase-minus cells is called senescence. Although senescing human somatic cells repress telomerase expression, the majority of tumors and immortalized cell lines reactivate telomerase. Interestingly, however, 10-15% of tumors can survive the absence of telomerase by maintaining their telomeres via an alternative pathway called ALT¹⁻³. Such alternative mechanisms could contribute to breast cancer as well, since 19-32% of early breast tumors and 5% of the advanced tumors were shown to be telomerase-minus⁴.

Telomerase-independent survival has also been observed in other organisms such as *Saccharomyces cerevisiae*⁵ and *Kluyveromyces lactis*⁶. The seminal characterization of telomerase-independent survivors of *S. cerevisiae* showed that *RAD52*-dependent recombination events could amplify telomeric and subtelomeric sequences and produce a functional telomere. A subset of *S. cerevisiae* as well as *K. lactis* survivors and ALT cells were shown to have greatly elongated telomeres, suggesting that commonalities in the mechanism of telomere maintenance may exist between these budding yeast survivors and human ALT cells. Since telomere maintenance is critical to oncogenesis, understanding both telomerase-dependent and telomerase-independent pathways will be important for therapeutic strategies.

We investigated the effect of mismatch repair (MMR) defects on telomerase-independent survival in *S. cerevisiae* and *K. lactis*. MMR has been shown to block recombination between imperfectly matched sequences⁷⁻⁹. Since telomeric and subtelomeric repeat sequences are not identical at different chromosome ends, we proposed that MMR could block recombination between such sequences and therefore repress telomerase-independent survival. Our results support this hypothesis and suggest the possibility that enhanced telomeric recombination in cells with MMR defects, including breast tissue¹⁰⁻¹⁵, may contribute to cell immortalization in the absence of telomerase reactivation during oncogenesis.

BODY

Methods

Yeast Strains

Gene disruptions were performed by standard yeast transformation and homologous recombination methods. The telomeric heterology was targeted to the telomere as described in¹⁶.

Growth Analysis

Serial streakout analysis: Growth phenotype was assessed by streaking for single colonies successively for up to 4X streakout from freshly dissected spores; successive streakouts were then reassembled on the same plate, growth properties were scored blind using an arbitrary scale of A(no growth) to E(wildtype-like growth). Whether growth of one mutant strain was better than another was determined by comparing the percent streakouts of each genotype showing the same growth characteristic at each successive streakout. For a subset of strains, at the point where successive streakouts were reassembled, cells from the colonies used for reassembly were resuspended in water, counted by hemacytometer and equivalent numbers of cells were placed in microtiter dishes. 10 fold dilutions were done in dishes and cells were stamped onto rich media plates. Dilutions and streakouts were growth for 2-3 days at 30°C. Results of dilution analysis and streakout scoring were similar.

Competition experiments: Cells from spore colonies of the desired genotypes were grown to log phase in rich media. Cultures were titered by hemacytometer and 10^4 cells/ml of two different strains were mixed together in 5 ml rich media and grown for ~21 hours (or until visible growth occurred in late senescing cultures). At the end of the growth period, cell counts were assessed by hemacytometer, ~1000 cells were plated for subsequent genotype determinations, the culture was diluted to 2×10^4 cells/ml and regrown. Percentage of viable cells of each strain in the culture was determined by replica plating onto the appropriate selective media.

Liquid growth assays: At least three independent samples of each strain were used. 5×10^5 cells from freshly dissected spore colonies were used to start 5 ml cultures in rich media. After ~21 hours of growth, cultures were titered by hemacytometer and diluted to 10^5 cells/ml. This was repeated for 9 days. Change in cell density over time was plotted. At each time point, the mean change in cell density of cultures of two different genotypes were compared, and the statistical significance of any difference in means was determined by the student's t-test. p values of 0.05 or lower were considered significant.

DNA preparations, Southern, Y' quantitation

Yeast genomic DNA preparations and Southern to quantitate Y' element levels were performed as previously described⁵. Phosphorimager quantitation was used to determine the signal intensity for the long and short Y' bands compared to an internal control band.

Results

MMR defects in S. cerevisiae enhance telomerase-independent survival in a RAD52-dependent manner

In *S. cerevisiae*, the mismatch repair protein Msh2 binds mismatches, either in complex with Msh3 or Msh6¹⁷. Msh3 and Msh6 have somewhat redundant functions in mismatch binding, such that Msh3 binds loop mismatches whereas Msh6 preferably binds to single-base mismatches^{18,19}. Mlh1-Pms1 heterodimer binds to the Msh/DNA complex in order to initiate mismatch repair. Mlh1 can also form heterodimers with Mlh2 or Mlh3, however, the Mlh1-Pms1 heterodimer appears to

play the most significant role in MMR²⁰⁻²³. Mutations in *MSH2*, *MLH1* or in *PMS1* have been shown to increase recombination between imperfectly matched sequences⁷⁻⁹. Mutations in *MSH3* and *MSH6* affect homeologous recombination differentially, depending on the nature of the heteroduplex formed and the assay system used^{9,24}. However, a double mutation in *MSH3* and *MSH6* enhance homeologous recombination to similar levels as does a mutation in *MSH2*⁹.

We tested the effect of mismatch repair mutations on telomerase-independent survival by comparing the growth phenotypes of multiple independent isolates of telomerase-minus strains to telomerase-minus strains that were also *msh2-Δ*, *mlh1-Δ*, *pms1-Δ*, *msh3-Δ*, *msh6-Δ*, or *msh3Δmsh6Δ*. Three different methods were used in order to assess growth differences between strains: 1) Comparative assessment of growth phenotype of successive single colony streakouts, 2) liquid competition experiments, 3) liquid growth assays. Collectively, our results show that *msh2-Δ*, *mlh1-Δ*, or *pms1-Δ* enhance telomerase-independent growth significantly (Figures 1 and 2). *msh3-Δ* and *msh6-Δ* did not exhibit detectable enhancement of telomerase-independent survival. However, a double mutation of *msh3-Δmsh6-Δ* enhanced survival significantly (Figure 1). Our results are consistent with the model that disruptions in these mismatch repair genes are enhancing telomerase-independent survival via an effect on homeologous telomeric and/or subtelomeric recombination.

RAD52-dependent recombination is required for propagating a telomerase-deficient strain i.e. for establishment of telomerase-independent survivors⁵. We have also shown that *RAD52* is needed for maintenance of such survivors (data not shown). We found that in the absence of *RAD52*, a telomerase-deficient strain could not be propagated, even when mismatch repair was defective (Figure 3). This shows that the enhancement of telomerase-independent survival by mismatch repair defects is through a recombination dependent pathway, and is not a complete bypass effect.

A proofreading defective POL3 does not enhance telomerase-independent survival
Defects in MMR increase mutation rates, as well as homeologous recombination rates. Therefore, we tested the possibility that just increasing the number of mutations in a telomerase-defective strain might enhance survival, presumably by introducing suppressor mutations. We found that a *pol3-01* mutation that renders DNA polymerase δ proofreading defective, thus producing mutation rates similar to that of *msh2-Δ*²⁵⁻³¹, has no effect on telomerase-independent survival (Figure 4). This result strongly suggests that it is not the increased number of mutations or the increased probability of acquiring suppressor mutations that enhance telomerase-independent survival in mismatch repair defective strains.

Defects in RAD1, RAD10, EXO1 do not affect telomerase-independent survival
The mismatch repair proteins Msh2 and Msh3, together with endonuclease components Rad1 and Rad10, are required for a specific type of repeat recombination, called Single Strand Annealing (SSA) when the process involves removal of non-homologous sequences^{32,33}. SSA can result in removal of intervening sequences when it occurs between tandem repeats. Since telomeric and subtelomeric regions contain numerous tandem repeats, it is conceivable that the telomerase-independent survival enhancement effect we observed in *msh2-Δ* could result, at least in part, from an inability to remove repeats by SSA (if such events occur), thereby stabilizing subtelomeric and telomeric repeat elements. We tested this by examining the effects of disrupting *RAD1* or *RAD10* on telomerase-independent survival and found that they have no effect on such survival (Figure 5). This supports the hypothesis that the role of *MSH2* in telomerase-independent survival is through effects on homeologous recombination, rather than SSA.

EXO1 is a 5' to 3' exonuclease that interacts with *MSH2*. Genetically, *EXO1* is in the same pathway as *MSH2*, possibly playing a role in the actual repair of mismatched DNA^{34,35}. *exo1-Δ* has a modest increase in mutation rate, and a small increase in homeologous recombination of certain substrates. We found that *exo1-Δ* has no effect on telomerase-independent survival. Therefore, downstream events required for repair of mismatches, such as the actual removal of mismatches, must be irrelevant to telomerase-independent survival. This is consistent with the role of *MSH2* in telomerase-independent survival being via an effect on homeologous recombination.

***MSH2* disruption in *Kluyveromyces lactis* enhances telomerase-independent survival**

S. cerevisiae telomeres consist of irregular G₁₋₃T repeats, whereas human telomerase adds a perfect T₂AG₃ repeat to chromosome ends. Therefore, we wanted to determine if the enhancement of telomerase-independent survival by mismatch repair defects was a phenomenon specific to a yeast with irregular telomeres or would also be observed in organisms where telomerase adds perfect telomeric repeats. Expecting to see such survival enhancement was reasonable, since it has been shown that the centromere-proximal region of the T₂AG₃ telomeric tract of human telomeres is somewhat degenerate, with potentially even higher levels of mismatches between subtelomeric regions³⁶⁻⁴⁰. These regions could provide a block to homeologous recombination via MMR. We investigated this in *K. lactis*, a budding yeast whose telomerase adds 25 bp perfect telomeric repeats to a G-rich telomere tract length that is approximately the same length as in *S. cerevisiae*.⁴¹ This yeast has a *RAD52*-dependent, telomerase-independent survival pathway that involves amplification of telomeric repeats, similar to *S. cerevisiae*⁶.

Since no *K. lactis* mismatch repair gene had been cloned, we first cloned the *klMSH2* gene. We used degenerate PCR primers in the well conserved ATPase domain of *MSH2* homologs to amplify the similar *K. lactis* genomic DNA region. Using this fragment as a probe we screened an *E. coli* cDNA library and identified a clone, that when sequenced showed 75% similarity and 59% identity to the *S. cerevisiae* *MSH2* gene at the amino acid level across the entire gene, lining up with previously identified, well conserved *MSH2* domains (Figure 6A). Disruption of *klMSH2*, replacing ~1kb mid-section of the *klMSH2* gene with *schIS3*, produces a ~100 fold increase in mutation rate, showing that *MSH2* function is obliterated by this disruption.

We then compared the growth of telomerase-minus strains with double mutant strains that were also *klmsh2-Δ*. In preliminary liquid competition experiments, the *klmsh2-Δ* telomerase-minus strain won the competition in 2/4, was represented at a higher proportion in 1/4, and was competed out by the *klMSH2* telomerase-minus strain in 1/4 of the cultures (Figure 6B); compared to 7/7 of *msh2-Δ* telomerase-minus strains winning the competition against telomerase-minus strains in *S. cerevisiae*. These initial results show that an MMR defect in *K. lactis* enhances telomerase-independent survival, albeit to a lesser extent than in *S. cerevisiae*. This suggests that MMR defects can enhance telomerase-independent survival even in an organism whose telomerase adds regular repeats, and strengthens the possibility that a similar enhancement in human cells may contribute to cell immortalization in human cells without reactivating telomerase.

MMR* deficiency causes a small increase in subtelomeric Y' repeat amplification of telomerase-minus *S. cerevisiae

We determined the levels of a certain type of subtelomeric repeat element, called the Y' element, in MMR deficient and proficient telomerase-minus strains at two successive time points during growth. We found that there is at most a two-fold increase in Y' levels in the absence of MMR (Table 1). Since Y' elements within a strain are ~99% identical⁴², it is possible that most of the survival enhancement effect observed in MMR deficient cells is due to a relief of a block to telomere-

telomere recombination, or recombination between less similar subtelomeric repeat elements, such as X elements or COS genes⁴³.

Development of a telomeric recombination assay for direct determination of changes in end recombination in the absence of MMR

We are currently developing an assay to measure the frequency with which a 4 nt sequence change introduced into a specific telomere marked with *URA3* moves to a Y' containing telomere (2/3 of telomeres are Y' containing). Movement of this heterology to a Y' telomere should be detectable by the appearance of a PCR product between a unique Y' sequence primer and a telomeric primer that preferentially anneals to telomeric sequences that have the heterology, but not to wildtype telomeric sequences.

We introduced a 4 nt change (BsgI site) into chromosome XIV-R in wildtype, *msh2-Δ*, *est2-Δ*, *est2-Δmsh2-Δ* strains in order to compare their telomeric recombination rates (Figure 7A). We worked out PCR conditions that give a PCR product with the telomeric primer that has the BsgI site but not with the telomeric primer that is wildtype (Figure 7B). We are currently working on testing whether we can observe telomere-telomere recombination in vivo, using PCR to monitor whether this 4nt change moves to other telomeres.

The effect of the complete absence of any subtelomeric sequences in a chromosome end

In an effort to better characterize factors that promote telomerase-independent survival, we tested whether the complete absence of subtelomeric repeats from both ends of a chromosome would decrease the frequency with which survivors are generated. We compared the growth of a telomerase-deficient strain in which the subtelomeric regions of chromosome III were deleted (using unpublished strains kindly provided by Dr. Jim Haber) to a strain with wildtype chromosomes, using serial streakout analysis. The complete absence of subtelomeric repeats from chromosome III has no detectable effect on telomerase-independent survival (data not shown). Since telomerase-deficient strains require *RAD52*-dependent recombination to maintain their telomeres, this result is consistent with telomere-telomere recombination between irregular, mismatched telomeric repeats being able to produce a functional telomere.

Key Research Accomplishments

-We showed that MMR defects enhance telomerase-independent survival in *S. cerevisiae* in a *RAD52*-dependent manner.

-We showed that increasing mutation rates to levels observed in *mmr*⁻ via a proofreading defective polymerase δ does not enhance telomerase-independent survival.

-We determined that MMR defects have very little effect on the levels of a subtelomeric repeat element that is 99% identical within a strain.

-We cloned and disrupted an MMR gene in *K. lactis*, a budding yeast with regular telomeric repeats, and preliminary data shows that an MMR defect enhances telomerase-independent survival in *K. lactis*, albeit to a lesser extent than in *S. cerevisiae*.

-We developed strains and optimized PCR conditions for a new telomeric recombination assay. We initiated experiments to test MMR proficient and deficient telomerase-minus strains for telomere-telomere recombination levels.

Reportable Outcomes

-Abstract and poster presentation: Yeast Genetics and Molecular Biology Meeting of the Genetics Society of America. University of Maryland, College Park. July 1998.

-Abstract, poster presentation, and invited short talk: FASEB Summer Conference. Genetic Recombination and Chromosome Rearrangements. Snowmass, Colorado. August 1999.

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Telomere maintenance in the absence of telomerase in *S. cerevisiae*. **Aylin Rizki, Victoria Lundblad.** Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.

Telomerase-deficient *S. cerevisiae* cells have progressively shorter telomeres, and once telomeres become too short to maintain function, the majority of the cells in the population stop dividing. We have initiated an analysis of the properties of single cells from early versus late telomerase-deficient cultures. Initial studies indicate that the lifespan of telomerase-minus cells is progressively reduced with continued propagation of the culture, whereas the lifespan of wild type cells remains constant. This suggests that telomerase-defective strains display progressive aging, in contrast to the fixed alteration in aging that occurs in mutant strains such as *sir4-42* or *sgs1* strains. We are currently asking whether there is a progressive increase in aging-related phenotypes, such as sterility or nucleolar fragmentation, in telomerase mutant strains. A second area of investigation focuses on the subpopulation of cells that can survive the eventual consequence of telomerase deficiency. Such 'survivors' undergo substantial amplification of both telomeric DNA as well as adjacent subtelomeric repeats. Both the formation and maintenance of survivor strains are dependent on *RAD52* function. We are currently testing the requirement for other components of the homologous recombination pathway, as well as determining the effects of mutations in genes involved in mismatch repair, single-strand annealing, direct-repeat recombination, and cellular lifespan.

Yeast Genetics and Molecular Biology Meeting of GSA. June 98

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Telomere Maintenance In The Absence Of Telomerase In *Saccharomyces cerevisiae*: Role of Mismatch Repair

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In eukaryotes, the ends of linear chromosomes are protected from damage and rearrangements by a complex of G-rich repetitive sequences and protein, called telomeres. Replication and maintenance of telomeres is necessary for normal cellular proliferation. In most eukaryotes, the reverse transcriptase telomerase is responsible for replicating the end of a chromosome and thus maintaining telomere length. Absence of telomerase activity results in progressive telomere shortening with continued cell division. When telomeres are too short to maintain function, most cells stop dividing. However, in *S. cerevisiae*, a subpopulation of cells can escape the lethal consequences of the absence of telomerase. This is not specific to budding yeast, as telomerase-independent survivors also have been observed in fission yeast and human cells.

In *S. cerevisiae*, survivors arise as a result of *RAD52*-mediated amplification of both telomeric and subtelomeric repeats. We have previously proposed that in the absence of telomerase, recombination can restore the G₁₋₃T telomeric repeats necessary for telomere function and thereby maintain cell survival. Since the terminal G-rich sequences are not perfectly homologous in *S. cerevisiae*, this could provide a potential barrier to recombination and therefore reduce the frequency with which survivors are generated. We have tested this, by demonstrating that mutations in genes in the mismatch repair pathway, previously shown to increase homeologous recombination frequencies, enhance telomerase-independent survival in a *RAD52*-dependent manner. Since little or no additional amplification of sub-telomeric repeats is observed in these survivors, this suggests that the absence of mismatch repair genes removes a block to G₁₋₃T-G₁₋₃T recombination. We are specifically testing this model by observing the effects of mismatch repair defects on the recombination behavior of individual telomeres in which the terminal G₁₋₃T tract has been marked by a very minimal sequence change. In addition, characterization of the effect of a mismatch repair defect on the frequency with which telomerase-defective survivors arise in *Kluyveromyces lactis*, a budding yeast that has perfectly homologous G-rich telomeric repeats, are under study.

Author Disclosure

- ☐ Check if there is a possible conflict of interest in presenting this information on the part of the author (s) or presenter, so that it may be noted in the Program.

Conclusions

We demonstrated that MMR defects in *S. cerevisiae* enhance telomerase-independent survival in a *RAD52*-dependent manner. Inability of a proofreading defective polymerase that increases mutation rates similar to *mmr*⁻ cells to enhance such survival suggests that the *mmr*⁻ effect is not a general mutator effect. We propose that increased homeologous recombination in telomeric and subtelomeric regions in *mmr*⁻ strains could enhance telomerase-independent survival. We are developing a telomeric recombination assay to test this more directly. We also found that this enhancement effect is not specific to a budding yeast with irregular telomeric repeats that provide a barrier to homeologous recombination. MMR defects in *K. lactis*, a yeast whose telomerase adds perfect telomeric repeats, also enhance telomerase-independent survival. This strengthens the possibility that MMR defects may increase chances of cell immortalization without re-activating telomerase via an effect on chromosome end recombination in human cells. Although human telomeres have perfect repeats, we propose that a similar enhancement effect may manifest itself as a removal of a block to homeologous recombination in the variable subtelomeric regions or the ~1kb of degenerate telomeric repeat region at the base of human telomeres.

Defects in the human homologues of *MSH2*, *MLH1* and *PMS1* genes have been shown to cause Human Hereditary Non-Polyposis Colon Cancer (HNPCC)⁴⁵⁻⁵¹. There is accumulating evidence that defects in these genes are present in some sporadic tumors as well, including some breast tumors¹⁰⁻¹⁵. If our results in budding yeasts prove to be relevant for human cell immortalization, this would have a tremendous effect on therapies based on inhibition of telomerase. If MMR defects in human cells enhance telomerase-independent cell growth via a boost in telomeric recombination, then inhibiting telomerase may not be an effective therapeutical method for treating *mmr*⁻ tumors.

We have pursued our proposed research goals diligently. However, following up on some very interesting results regarding the mismatch repair effect, we have made some modifications to our actual plan, or SOW, as described below:

Specific Aim 1: Determination of the role of subtelomeric sequences and telomeric recombination in telomere maintenance in the absence of telomerase

Previous (1A) and (1B) Monitor the behaviour in *S. cerevisiae* of an artificial subtelomeric repeat

Modified (1A) and (1B) Determine the levels of the Y' subtelomeric repeat element in MMR deficient and proficient telomerase-minus strains

Tasks 1-9 (Modified-see Body): Months 1-9: COMPLETED

Previous (1C) Convert *K. lactis* yeast to a strain that has artificial subtelomeric repeats

Modified (1C) Determine the effect of a mismatch repair defect on telomerase-independent survival in *K. lactis*

Tasks 10-13 (Modified-see Body): Months 10-13: COMPLETED

(1D) Determine the effect of the complete absence of any subtelomeric sequences in a chromosome end

Tasks 14-17: Months 8-12: COMPLETED

(1E) Determine the frequency of telomeric recombination between G-rich repeats

Tasks 18-20: Months 13-17: COMPLETED

Task 21: Months 18-20: IN PROGRESS

Specific Aim 2: Determination of the genetic factors involved in the establishment, maintenance, and frequency of occurrence of survivors

(2A) Determine if recombination is required for the maintenance of survivors

Tasks 22,23: Months 19-20: COMPLETED

(2B) Determine the effect of mismatch repair gene mutations on the frequency of survivor formation in *S. cerevisiae*

Tasks 24-26: Months 24-26: COMPLETED

Task 27: Months 25-27: IN PROGRESS

Previous (2C) A mutant screen for mutations that increase the frequency of survivors

Modified (2C) A candidate gene approach to determination of factors that are important for telomerase-independent survival

Task 28: Months 27-30: Determine if a DNA damage checkpoint response is involved in promoting recombination events required for telomerase-independent survival, by observing the effect of checkpoint gene mutations on telomerase-independent survival.

Task 29: Months 30-33: Determine if factors, such as the *Ku70/Ku80* or *CDC13*, that have been proposed to protect chromosome ends from being recognized as damage are no longer at the telomere, allowing recombination events to occur at a much higher frequency in the absence of telomerase, by in vivo formaldehyde crosslinking analysis.

Task 30: Months 33-36. Determine if mechanisms that block recombination in wildtype cells, e.g. MMR, are suppressed in telomerase-deficient cells. Test if there is an increase in mutation rates, double strand breaks, recombination intermediates (Chi structures), and single strandedness in telomeric regions as an indication of such suppression.

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Figure Legends

Figure 1. Effect of *mmr-Δ* mutations on the serial streakout growth of telomerase-defective (*est2-Δ*) strains

Multiple independent spore colonies of each genotype were serially streaked out to 4X. The number of samples of each genotype is indicated in parenthesis e.g. *est2-Δmsh2-Δ*(18). The level of growth of each streakout was scored as described in Methods. Each graph shows the distribution of growth scores of each genotype for each streakout point. e.g. 100% of the 18 *est2-Δmsh2-Δ* 1X streakouts were given a growth score of E (wildtype-like growth). Going from left to right of each row, graphs represent growth distribution of 1x, 2x, 3x, and 4x streakouts.

Figure 2. Effect of *mmr-Δ* mutations on growth rates of telomerase-deficient strains in liquid competition experiments

Equal numbers of cells from spore colonies of two different strains (e.g. *est2-Δ* and *est2-Δmsh2-Δ*) were grown together in culture. Each culture was propagated and the percentage of viable cells of each strain in culture were determined as described in Methods. Graphs of %viable cells of each genotype was plotted against time in culture. As a control, the effect of *mmr-Δ* mutations on telomerase-proficient cells was also determined (e.g. wt vs. *msh2-Δ*).

Figure 3. Effect of *rad52-Δ* on *mmr-Δ* telomerase-deficient strains

Multiple independent spore colonies of the indicated genotypes were streaked out and their growth was compared as described in Methods. This figure shows an example of the results of these experiments, showing growth at 1x (25 generations) and 2x(50 generations) streakouts. Multiple samples from *msh2-Δ*, *mlh1-Δ*, *pms1-Δ*, *msh3-Δ*, *msh6-Δ*, and *msh3-msh6-Δ* strains were tested similarly. Results were comparable that shown for one *msh2-Δ* sample in this figure.

Figure 4. Effect of *pol3-01* on telomerase-independent survival

(A) Three independent samples of each genotype were subjected to liquid growth experiments as described in Methods. Each point on this graph shows the mean value of the number of cells in three cultures of the same genotype at each time point. The numbers in parenthesis are p-values calculated by Student's t-test, comparing the *est-Δ* to the *pol3-01est2-Δ* strains at the indicated time point. $p \leq 0.05$ reflects a significant difference between the compared data sets. (B) Serial streakout assessment of growth, as described for Figure 1.

Figure 5. Effect of *rad1-Δ*, *rad10Δ*, and *exo1-Δ* mutations on the serial streakout growth of telomerase-defective strains

Serial streakout assessment of growth, as described for Figure 1.

Figure 6. Effect of *K. lactis msh2-Δ* mutation on telomerase-independent (*ter1-Δ*) growth

(A) Amino acid sequence alignment of *S. cerevisiae* and *K.lactis* MSH2 genes, using ClustalW and Boxshade. Black shading shows identity, grey shading shows similarity between amino acids. (B) Liquid competition experiments, as described for Figure 2.

Figure 7. Telomeric Recombination Assay

(A) Schematic representation of chromosome XIV-R end in strains which have the 4 nt change (Bsg1 site) targeted to the telomere. A URA3 marker was used to select for targeting. The Bsg1-telomeric primer has a 4 nt change in its 3' terminus, compared to a wildtype telomeric primer. (B) In strains that have the Bsg1 targeted to chr XIV-R, PCR reactions using a forward URA3 or Y' primer and a reverse wildtype- or Bsg1-telomeric primer are expected to give the results indicated. As a negative control, a strain that does not have the targeted change at its chr XIV-R was used in parallel PCR reactions.

Figure 1.

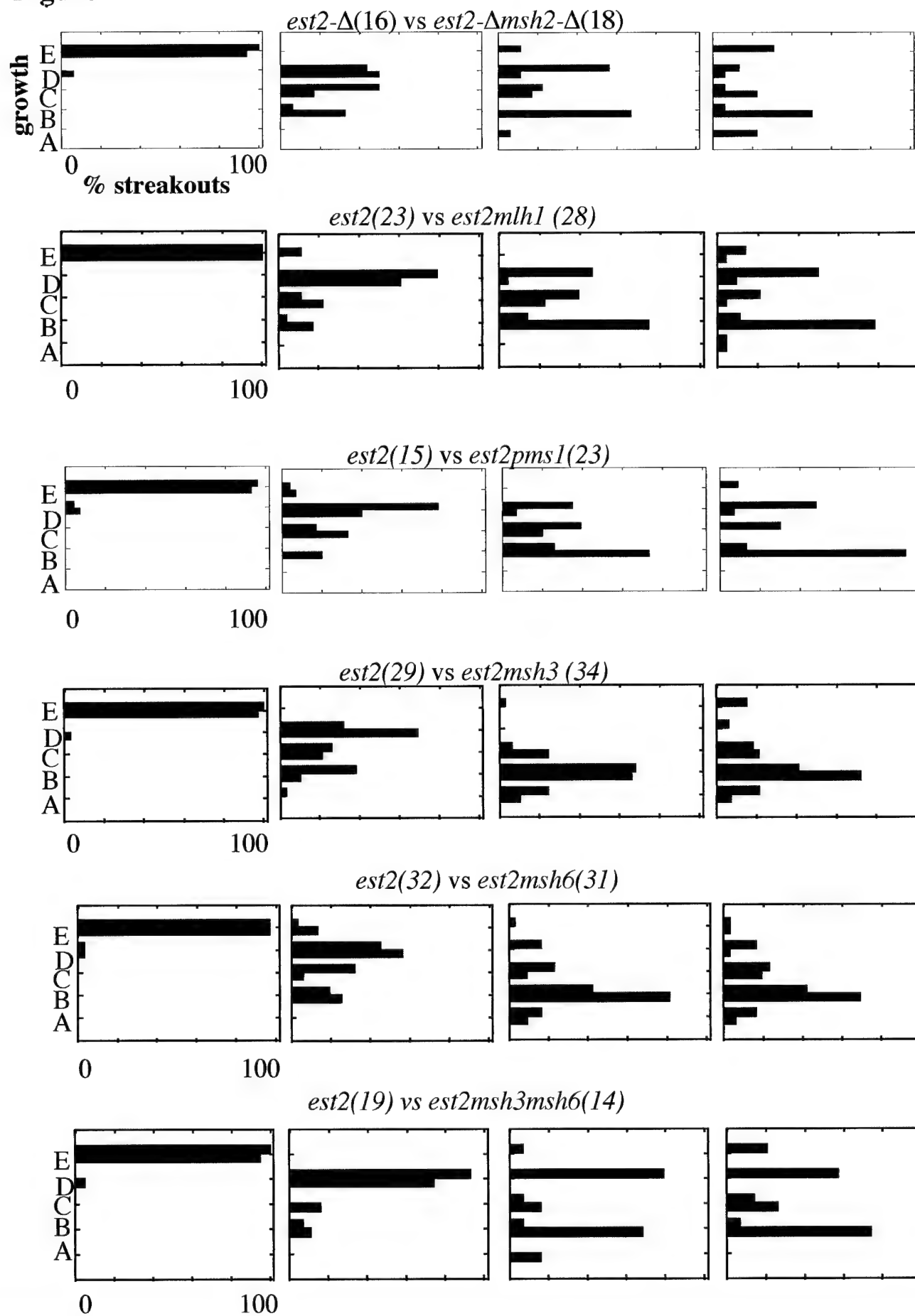


Figure 2.

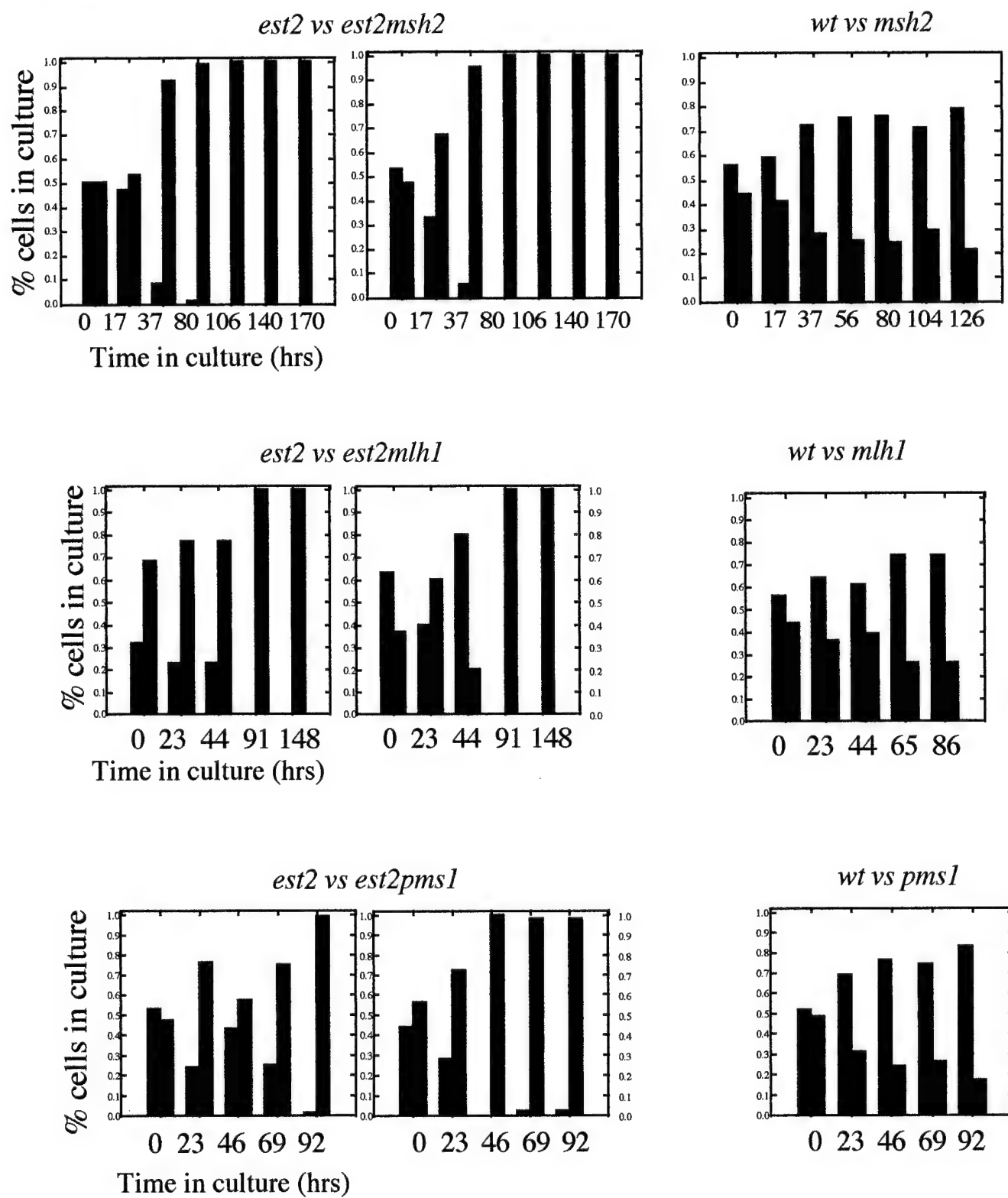


Figure 3.

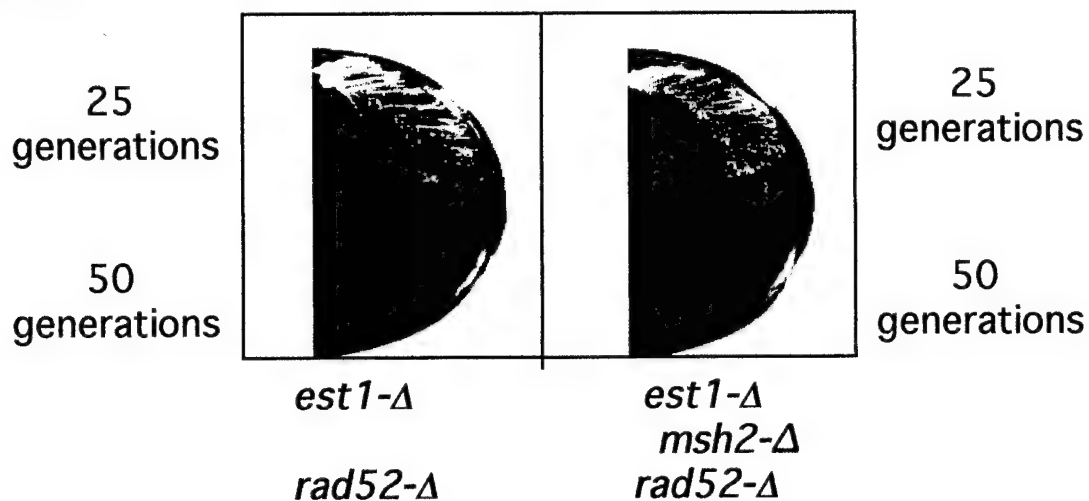
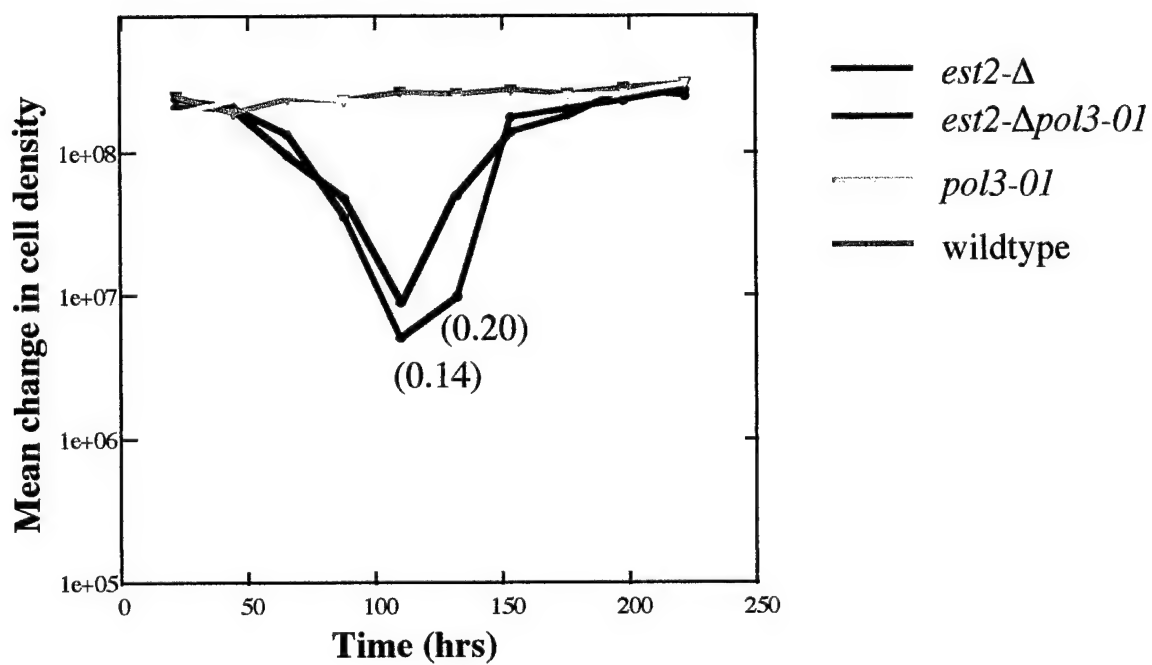


Figure 4.

A.



B.

est2-Δ (16) vs *est2-Δpol3-01*(18)

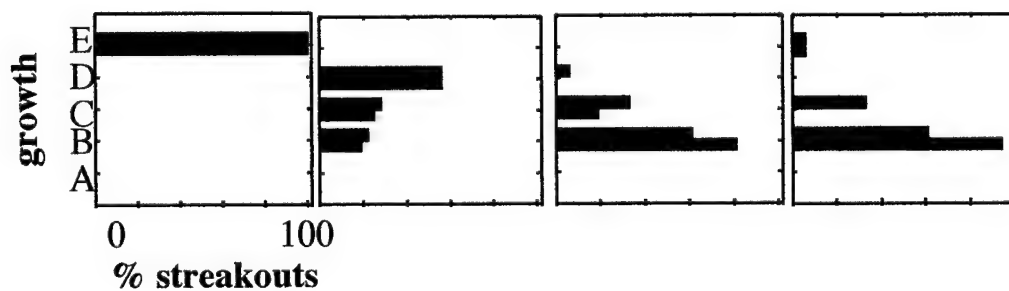


Figure 5.

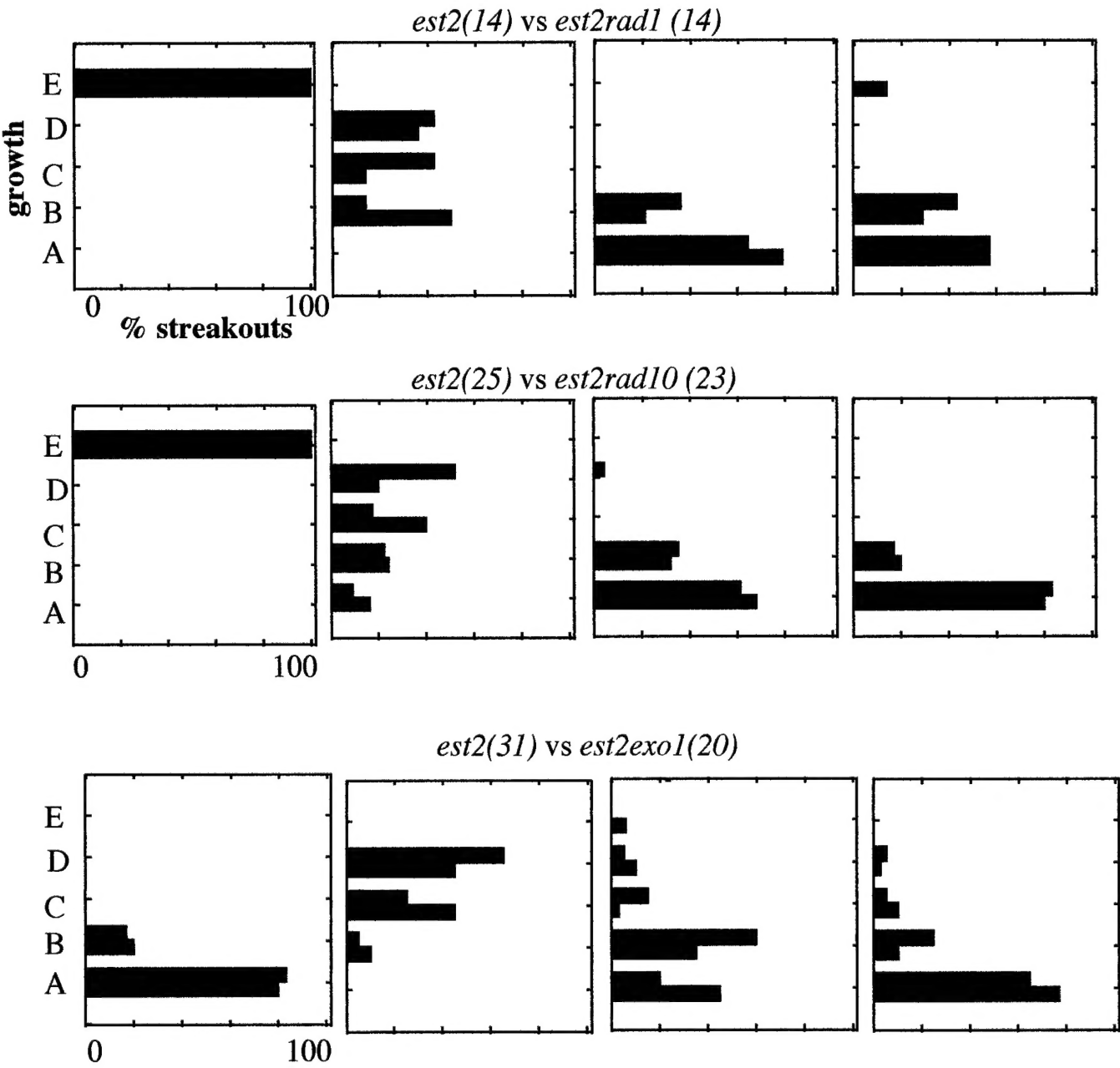


Figure 6.

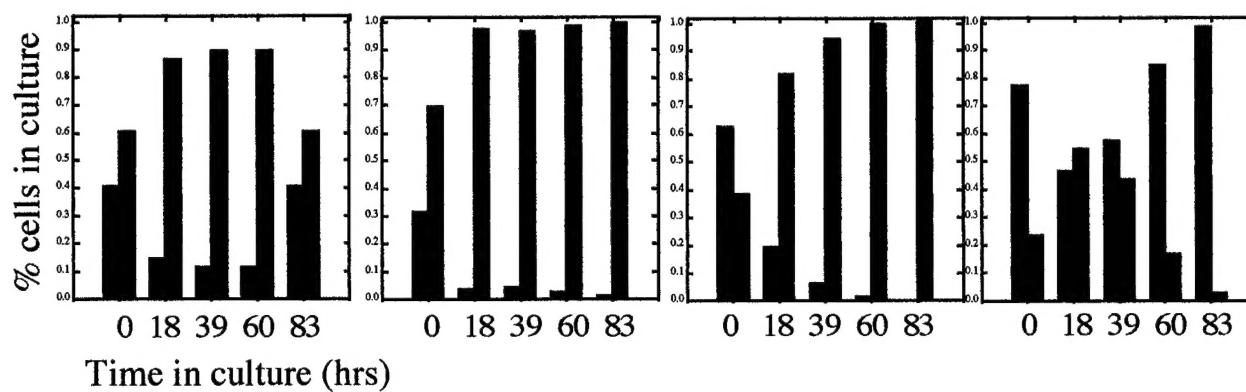
A.

<i>K.lactis</i>	1	GRPLDACSRFTILQRSEDSQEVFFITSQRS--QNTIRLDESDYITVVGDDALFVADQVSHD
<i>S.cerevisiae</i>	1	MSSTRPELKGSDVSEERNFYKKTGLPKKPLKTIRLVDEGDYITVIGSDAIFVADSVTHT
<i>K.lactis</i>	59	SVLEDCCKIDLANFANFDLOEPLKYITMSIQIYGNLLETSTLELGKKIEIYDRWKLKLT
<i>S.cerevisiae</i>	61	QSVLEKNCQLDPYTA--NFMETPKYITVSLQVLATLLEKLCGLDLGYEVEIYDKGKLIKLS
<i>K.lactis</i>	119	ASPGNLEQVDDLIYGSYETNVLASLKLFLGTAASTICTGVSFVDNTRYRIGLFDPLDN
<i>S.cerevisiae</i>	119	ASPGNIEQVNEHNNHNISSIIASLKVQWL-SQDGLCIIGVAFIDTAYKVGMLDIVDN
<i>K.lactis</i>	179	EVFSNLESCLIQLGKECLIPDLRDP SHANDLEKILSYIDRCSTVVSFYKPSDFNGKDY
<i>S.cerevisiae</i>	178	EVYSNLESFLIQLGVECLVQDLTSSNSNAHQKVIYIDRCGCYITLLKSEFSEKDY
<i>K.lactis</i>	239	EADIAKLGGDELSLSYS-RFSANCLGACSVLLIVNIMHNEANVGNFEVVDHSLSQFVKL
<i>S.cerevisiae</i>	238	ELDLTKLLGDDLALSLPQKYSKLSHGACHALIGTLQLLSEQDQVGKTELVEHKLKEPCKL
<i>K.lactis</i>	298	DASAIKALNVFPPTGSQG-----NTALLSVSSPQKCSSLQOLLNRCKTNSGVRLLNEV
<i>S.cerevisiae</i>	298	DASAIKALMLFPQGPQMPFGSNLAVSGFTSAGNSGKVTSLFQOLLNCKTNAAGVRLLNEV
<i>K.lactis</i>	350	LKQPLTDIDQITKRHDLVEFFDQLELRSLSQEECLPSYDIDRRLTKELQKNGHLEDVLE
<i>S.cerevisiae</i>	358	LKQPLTNIDEIKKRHDLVDYLDQIELRQMLTSEYLPMTPIDRRLTKELKRGHLEDVLE
<i>K.lactis</i>	410	IYQFAQHVPIVSDLLKQYEEVD-SNDLMILVKEVLIDDPHLENAILKELKELVEPTVYI
<i>S.cerevisiae</i>	418	IYQFSKRIPETVQVFTSFLBODSPTEPVNELVRSVWLAQLSHHVEPLSKFEEMVETTVDL
<i>K.lactis</i>	469	EAYEETNEFMKTEFELIQLSIIRTYLDELEDAIRYTHLDTADDLGFDPEKKLKLKELHLH
<i>S.cerevisiae</i>	478	DAYEENNEFMKTEFEEELGKIRSKLDTLRDEIHSIHLDSAEDLGFDPEKKLKLKELHLH
<i>K.lactis</i>	529	GVCHRLTRNDAAKALRQHKYIELSTVKAGIFFSTELKNTAEETSSELQKYEQQQASLYK
<i>S.cerevisiae</i>	538	GVCHRLTRNDAAKELRQHKYIELSTVKAGIFFSTELKESIANETNILEQKEYDEQQSALYR
<i>K.lactis</i>	589	EIVSITLSYTPVLEKLSVYTAQLDILCSFAQVSSYAPIPYVRPEHYPLNDQRTTELTA
<i>S.cerevisiae</i>	598	EINNTLTLYTPVFEKLSVYLAHLVDIASFARTSYAPIPYVRPEKLPMDSE-RRTDLISS
<i>K.lactis</i>	649	RHPIVEHQDDYTFISNDVKLYQGDSEFIYITGPMHGGKSTIIRQIGVICLMAQIGCFVPC
<i>S.cerevisiae</i>	657	RHPVLEHQDDISFISNDVLESGKGDFLIITGPMHGGKSTIIRQVGVISLMAQIGCFVPC
<i>K.lactis</i>	709	DEAKTAAVDAILCRVGAGDSQLKGVSTFMHEMLETASILKNATNNSLVIYDELGRGTSTY
<i>S.cerevisiae</i>	717	EEAEIATVDAILCRVGAGDSQLKGVSTFMVEILETASILKNASKNLSLVIYDELGRGTSTY
<i>K.lactis</i>	769	DGFGLTWSISENIATMLNCFILFATHFHELTTLADKLDTVSNHHIVAHIEDGSGS---HNS
<i>S.cerevisiae</i>	777	DGFGLAWAIAEHIASKLGCFAIFATHFHELTLESEKLPVYKNNHIVAHIEKELKEQKHDD
<i>K.lactis</i>	826	DDITLLYKVEAGSSDQSGIHVAEVVQFPKIVMAKREAAELEDLEKDDSLKKAKLSL
<i>S.cerevisiae</i>	837	EDITLLYKVEPGISDQSGIHVAEVVQFPEKIVMAKREANELDDLKTNNEEDLEKAKLSL
<i>K.lactis</i>	886	EDIVLGTESLEKSLKLVCKELKKNHLIDRLDDPSYQEEGVDELKQIDHLAREEDTTSLF
<i>S.cerevisiae</i>	897	QEVNEGNIIRLKLALKEVIRKYNEEGLHD--PSKITEEASQNEIQELLRAIAEPEKENDN
<i>K.lactis</i>	946	VDWYRDTLL-
<i>S.cerevisiae</i>	955	YLYIKALLL

Figure 6.

B.

ter1-Δ vs *ter1-Δmsh2-Δ*



wt vs *msh2-Δ*

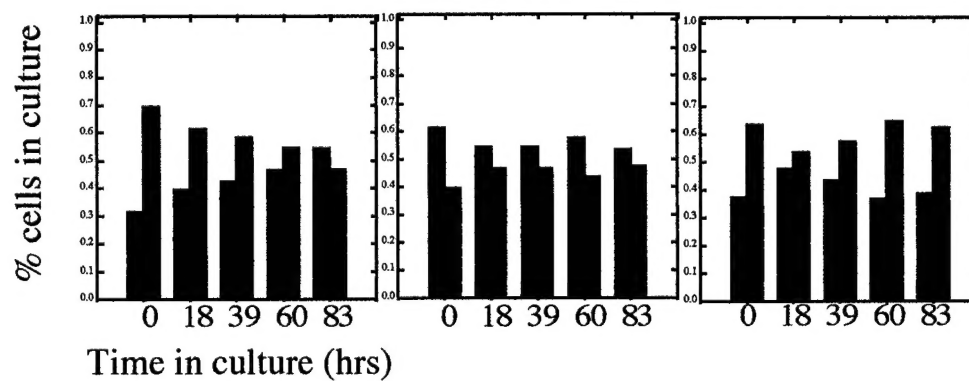


Table 1.

Genotype	Normalized Y' level , +/-SD, (#samples)	
	From 1x colonies	From 2x colonies
<i>est1-Δ</i>	4.8 +/-3.1 (19)	10.7 +/-3.1 (19)
<i>est1-Δ msh2-Δ</i>	7.3 +/-3.1 (22)	14.2 +/-6.4 (22)
<i>est2-Δ</i>	4.4 +/-3.3 (24)	8.8 +/-4.7 (24)
<i>est2-Δ mlh1-Δ</i>	5.4 +/-3.3 (23)	12.2 +/-17.7 (21)
<i>est2-Δ</i>	2.3 +/-1.0 (11)	2.8 +/-0.8 (11)
<i>est2-Δ pms1-Δ</i>	3.1 +/-1.4 (8)	5.4 +/-3.8 (8)

Figure 7.

A.



B.

forward primer	reverse primer	if site does NOT move	if site moves
----->	<-----		
URA3	wiltype telomeric	+	+
URA3	BsgI-telomeric	+	+
Y'	wildtype telomeric	+	+
Y'	BsgI-telomeric	-	+